THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

# Ketamine enhances structural plasticity in mouse mesencephalic and human iPSC-derived dopaminergic neurons via AMPAR-driven BDNF and mTOR signaling

**Citation for published version:**
Cavalleri, L, Merlo Pich, E, Millan, MJ, Chiamulera, C, Kunath, T, Spano, PF & Collo, G 2017, 'Ketamine enhances structural plasticity in mouse mesencephalic and human iPSC-derived dopaminergic neurons via AMPAR-driven BDNF and mTOR signaling', *Molecular Psychiatry*, vol. 23, no. 4, pp. 812-823. https://doi.org/10.1038/mp.2017.241

**Digital Object Identifier (DOI):**
10.1038/mp.2017.241

**Link:**
Link to publication record in Edinburgh Research Explorer

**Document Version:**
Peer reviewed version

**Published In:**
Molecular Psychiatry

**General rights**
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

OPEN ACCESS

Download date: 06. May. 2023

# Ketamine enhances structural plasticity in mouse-mesencephalic and human iPSC-derived dopaminergic neurons *via* AMPAR-driven BDNF and mTOR signaling

Laura Cavalleri MBS[1], Emilio Merlo Pich MD[2], Mark J. Millan PhD[3], Cristiano Chiamulera PharmD, PostGrad MSc[4], Tilo Kunath PhD[5], PierFranco Spano PhD[1] and Ginetta Collo MD[1,6]

[1]Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy.

[2]CNS Therapeutic Area Unit, Takeda Development Center Europe, London, UK.

[3]Division of Psychopharmacology, Institut de Recherches Servier, Croissy-Sur-Seine, France.

[4]Department Public Health & Community Medicine, University of Verona, Verona, Italy.

[5]MRC Centre for Regenerative Medicine, Institute for Stem Cell Research, University of Edinburgh, Edinburgh, UK.

[6]Department of Biomedicine, University of Basel, Basel, Switzerland.

Correspondence should be addressed to Ginetta Collo, Department of Molecular and Translational Medicine, University of Brescia, Viale Europa 11, 25123 Brescia Italy.

Telephone: +39-030-3717525; FAX: +39-030-3717529.

Email: (collo@med.unibs.it)

Running title: Ketamine mTOR-driven plasticity in DA neurons

**Abstract**

Amongst neurobiological mechanisms underlying antidepressant properties of ketamine, structural remodeling of prefrontal and hippocampal neurons has been proposed as critical. The suggested molecular mechanism involves downstream activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors which trigger mammalian target of rapamycin (mTOR)-dependent structural plasticity *via* Brain Derived Neurotrophic Factor (BDNF) and protein neo-synthesis. We evaluated whether ketamine elicits similar molecular events in dopaminergic (DA) neurons, known to be affected in mood disorders, using a novel, translational strategy that involved mouse mesencephalic and human induced pluripotent stem cells (iPSCs) derived DA neurons. Sixty minutes exposure to ketamine elicited concentration-dependent increases of dendritic arborisation and soma size in both mouse and human cultures as measured 72 hours after application. These structural effects were blocked by the mTOR complex/signaling inhibitors like rapamycin. Direct evidence of mTOR activation by ketamine was revealed by its induction of p70S6 kinase. All effects of ketamine were abolished by AMPA receptor antagonists and mimicked by the AMPA positive allosteric modulator CX614. Inhibition of BDNF signaling prevented induction of structural plasticity by ketamine or CX614. Furthermore, the actions of ketamine required functionally-intact dopamine D3 receptors (D3R), since its effects were abolished by selective D3R antagonists and absent in D3R knockout preparations. Finally, the ketamine metabolite 2R,6R-hydroxynorketamine mimicked ketamine effects at sub-micromolar concentrations. These data indicate that ketamine elicits structural plasticity in DA neurons by recruitment of AMPAR, mTOR and BDNF signaling in both mouse mesencephalic

and human iPSC-derived DA neurons, observations of likely relevance to its influence upon mood and its other functional actions *in vivo*.

## Introduction

Ketamine is a dissociative general anaesthetic that acts as a non-competitive antagonist at the N-methyl-D-aspartate glutamate receptor (NMDAR).[1] At sub-anesthetic doses, ketamine displays a complex psychoactive profile reflecting at least partially its influence on central glutamatergic and monoaminergic neurotransmission.[2,3] Importantly, recent work has shown that a single infusion of low dose ketamine over 40-60 minutes in subjects with major depression produces rapid antidepressant effects (within hours) that persist for up to 1-2 weeks, well beyond the duration of its initial psychotomimetic effects or pharmacokinetic exposure.[3,4,5] A similar profile was observed in rodents.[5,6,7]

While molecular substrates underlying the antidepressant effects of ketamine still remain unclear, studies on rodent models offered important mechanistic insights. Converging findings suggest that ketamine enhances glutamatergic neurotransmission in frontocortical and hippocampal pyramidal neurons, possibly reflecting attenuation of the inhibitory input from local GABAergic interneurons onto glutamatergic neurons[6,8,9] and/or a reduced constitutive glutamatergic control of excitatory synaptic drive at pyramidal neurons.[8,10] The increase in synaptic glutamate neurotransmission is thought to preferentially activate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR)[8,11,12] that promote synthesis and release of Brain Derived Neurotrophic Factor (BDNF),[13,14] which in turn drives structural plasticity mediated by protein synthesis and dendrite outgrowth *via* activation of mammalian target of rapamycin (mTOR) signaling.[15,16,17]

Defective functional and structural plasticity in glutamatergic frontocortical/hippocampal circuits and/or mTOR downregulation has been described in patients with mood disorders[18,19,20] and in rodents after chronic stress.[21,22] These phenomena were reversed by chronic electroconvulsive therapy, chronic pharmacological treatment with serotonin uptake inhibitors (SSRIs)[23,24,25] and ketamine infusion.[15] Their antidepressant actions involve activation of BDNF/TrkB signaling and protein neosynthesis, producing incremental structural plasticity.[23,24,25,26] Structural plasticity can be defined and characterized by evidence for morphological changes in primary dendrite length, dendrite number and/or branching, an increased soma area[27] and/or changes in the number and shape of dendritic spines.[15]

However, engagement of frontocortical and hippocampal circuits may not fully explain the antidepressant properties of ketamine. For example, anhedonia, a core symptom of major depression, has been consistently associated with dysfunctional mesolimbic dopaminergic (DA) transmission.[28,29] The role of the DA system in depression is supported by the evidence of reduced levels of dopamine transporter (DAT) in basal ganglia[30] and, indirectly, by the antidepressant properties of drugs that activate dopamine neurotransmission, such as nomifensine, bupropione and amphetamine.[31] Significant antidepressant effects can be also produced by pramipexole, a DA agonist with preferential affinity for the dopamine D3 receptor (D3R).[32,33,34] D3Rs are enriched in ventral mesencephalon and expressed in DA neurons.[35,36,37] Recent experiments showed that D3R-preferential DA agonists and drugs that stimulate DA neurotransmission such as amphetamine and cocaine can produce structural plasticity *in vitro* in mouse mesencephalic DA neurons.[38,39] These effects are mediated by activation of D3R and its intracellular pathway which includes

mTOR signaling.[40] Interestingly, ketamine has been shown to enhance DA neurotransmission in rodents and humans,[41,42,43] to possess acute psychomimetic effects[2] and to have addictive potential[1] in addition to its alleviation of anhedonia and depression: all these actions might be related to an influence upon the functional plasticity of DA neurons.[44]

In light of the above, the present study directly examined the molecular substrates underlying the influence of ketamine upon structural plasticity in DA neurons *in vitro* as defined by maximal dendrite length, primary dendrite number and soma area.[27] To this end, we adopted a translational cellular approach that incorporated both mouse and human cellular models of DA neurons. The mouse procedure was based on an established protocol of primary mesencephalic DA neurons,[38,39,40] while the human model employed human inducible pluripotent stem cells (iPSCs) derived from a healthy donor and differentiated into DA neurons.[45,46] The latter methodology is a relatively recent development in neuropharmacology but increasingly recognized as a translationally-relevant approach of importance for the characterization of potential therapeutic agents.[47]

## Materials and methods

A more detailed description is found in Supplementary Materials and Methods and in Supplementary Tables.

*Pharmacological agents*

Pharmacological agents used in the present study are detailed in Supplementary Table S1. For each vehicle treatment, solvents required by specific drugs were used at the same dilution used for the active treatment.

*Animals*

CD1 mice were provided by Charles River Laboratories (Calco, Italy); D3R knockout (D3KO)[48] and C57BL6/J syngenic mice by Jackson Laboratory (B6.129S4-Drd3Tm1Dac/J). Animal care was in accordance with the European Community Council Directive of September 2010 (2010/63/EU) with the approval of the Institutional Animal Care and Use Committee of the University of Brescia and in line with the Italian law.

*Mouse primary mesencephalic cultures*

Mouse mesencephalic neurons from embryonic day (E) 12.5 were prepared as previously described.[38,39,40]

*Human iPSCs culture and dopaminergic differentiation*

NAS2 human iPSCs[45] were cultured on Matrigel-coated (BD Biosciences, San Jose, CA) plates in mTeSR™1 medium (StemCell Technologies, Vancouver, BC, Canada).

Floor-plate based midbrain DA neuron induction and dual-SMAD inhibition protocols were performed.[46]

*In vitro pharmacological experiments: structural plasticity.*

Neuronal cultures grown on coated coverslides were exposed to 0.001-10 µM ketamine for 60 min. At the end of treatment the medium was replaced and neurons were maintained in culture until fixation that was performed at 72 hrs.[40] Other compounds (7-OH-DPAT,[38] Ro 25-6981,[49] CX614,[50] (2R,6R) hydroxynorketamine (2R,6R)-HNK[51]) were used at concentrations ranging from 0.001 to 10 µM (Supplementary Table S1) and applied for at least 12 hrs. Pharmacological inhibitors and receptor antagonists were added to the cultures 20 min prior treatments. Fixed cultures were stained with an anti-tyrosine hydroxylase (TH) antibody to identify DA neurons and morphometrical analysis was performed to assess maximal dendrite length, primary dendrite number and soma area.[27,38,39,40] The sample size per condition was 30, 50 and 40, respectively, obtained from two coverslides per treatment group.

*In vitro pharmacological experiments: intracellular signaling.*

The rapid effects of ketamine on intracellular signaling were assessed by western blot and immunofluorescence. The time-response curve of phosphorylated p70S6K (p-p70S6K) to 1 µM ketamine was obtained at 2, 5, 10, 30 and 60 min by western blot. Pharmacological inhibitors and receptor antagonists were added to the cultures 20 min prior treatment with ketamine and analyzed at 5 min after exposure, when the p-p70S6K signal was maximal. For studies comparing mesencephalic cultures from D3KO and wild-type mice, cultures were exposed to either 1 µM ketamine or 50 nM

7-OH-DPAT for 5 min. The sample size per condition for western blot was 4-6, each data being biological replicate from different experiments; for immunofluorescence was 30 for $TH^+$ neurons and 60 for $TH^-$ neurons, measured from two coverslides per treatment group.

*Statistical analysis*

All statistical analyses were performed on biological replicates. Data were expressed as mean ± standard error of the mean (S.E.M.), if not stated otherwise. Measurements of structural plasticity parameters, western blot and immunofluorescence were tested for normality (D'Agostino & Pearson), either as crude data or after log transformations. Significant differences from control conditions were determined using either two-tailed unpaired Student's *t*-test, one-way or two-way analysis of variance (ANOVA) followed by *a posteriori* Bonferroni's test for multiple comparisons. When the test of normality was not satisfied, non-parametric tests were used (e.g., Kruskal-Wallis or Friedman) followed by *a posteriori* Dunn's test. The sample size for assessing structural plasticity was based on available data[39,40] to detect a medium-large effect size (d=0.4-0.6), with alpha = 0.05 and power 80%. Each experiment was replicated at least twice. All data were processed with Prism - version 7.0 (GraphPad Software, San Diego, CA). Outlier values were excluded if > 2 S.D. from the mean.

**Results**

*Structural plasticity induced by ketamine in primary cultures of mesencephalic DA neurons depends on the activation of PI3K-mTOR signaling.*

We studied the effects of ketamine on DA neurons in primary cultures generated from

E12.5 mouse mesencephalon. DA neurons were visualized using anti-TH antibodies ($TH^+$) (Figure 1a and Supplementary Figure S1a); they represented 10%±4% of neurons co-stained with anti-MAP2 antibody, these $MAP2^+$ neurons constituting 90-95% of the total number of cells in the culture. Dual immunofluorescence analysis showed that $TH^+$ neurons co-expressed the dopamine transporter (DAT), confirming the phenotypic maturity of DA neurons (Supplementary Figure S1b). GABAergic ($GAD67^+$) and glutamatergic ($VGLUT2^+$) neurons were also identified (Supplementary Figures S1c and d), representing 20%±7% and 38%±9% of neurons, respectively. Both DA neurons and GABAergic neurons co-expressed NMDA/$NR2B^+$ puncta (Supplementary Figure S1e and S1f).

Neuronal cultures were exposed to ketamine at a low concentration (micromolar range) for 60 min, i.e., conditions that approximately match ketamine use *in vivo*.[1,2,3,6] After the initial exposure, the culture medium was washed-out and replaced by ketamine-free medium. Cultures were fixed at 72 hrs and processed for assessment of structural plasticity. Significant concentration-dependent (0.001-10 μM) increases of dendritic arborization and soma size were observed in $TH^+$ neurons (Figure 1a-b and Supplementary Figure S2). Since a reliable significant effect was observed at 1 μM, this was the dose used in all the following experiments. Validation of the actual concentration in media was performed using HPLC-mass spectrometry that showed levels of 0.9±0.1 μM very close to the original concentration applied (1 μM). Further, no ketamine metabolites were detected following incubation.

Pretreatment with the PI3K inhibitor LY294002 (10 μM) or the mTORC1 inhibitor rapamycin (20 nM) completely blocked the effects of ketamine on all canonical parameters used to assess structural plasticity (Figure 1c). The involvement of the PI3K-mTOR pathway in the effects of ketamine was further confirmed in acute

phosphorylation experiments performed at 2, 5, 10, 30 and 60 min exposure. Using western blot, we found that ketamine increased mTORC1-dependent p70S6K phosphorylation (p-p70S6K) with a maximal effect at 5 min, persisting for 60 min (Figure 1d). This ketamine effect was antagonized by pretreatment with LY294002 (10 μM) (Figure 1e). Parallel immunofluorescence experiments showed that ketamine significantly increased the basal levels of p-p70S6K in $TH^+$ neurons, demonstrating that they at least partially account for the observations in the western blot studies (Figure 1f and g). In addition, ketamine induced a p-p70S6K signal in $TH^-$ neurons suggesting that they may also be involved. Ketamine effects on p-p70S6K were antagonized by pretreatment with LY294002 (10 μM) (Figure 1f and g). Overall, these data indicate that the PI3K-mTOR signaling pathway is engaged in the influence of ketamine on mouse mesencephalic DA neurons and may well be involved in its effects on structural plasticity.

*AMPA receptors coupled to mTOR signaling are involved in ketamine-induced structural plasticity.*

Using double-staining immunofluorescence we found that both GluR1 and GluR2 AMPAR subunits were expressed in $TH^+$ neurons in the mouse mesencephalic cultures (Figures 2a and b), in line with published data.[52] Semiquantitative immunofluorescence image analysis indicated that GluR1 was preferentially expressed in the soma while GluR2 clustered in dendrites (Figure 2c). Since AMPAR activation is known to trigger protein synthesis and dendrite outgrowth in neurons,[13,53,54] we exposed the cultures to the AMPAR positive allosteric modulator (PAM) CX614 enhancing structural plasticity in a concentration-dependent fashion (0.5-10 μM) (Supplementary Figure S3b). The effects of CX614 (10 μM) were

blocked by pretreatment with the selective AMPAR antagonist NBQX (10 μM) and also by rapamycin (20 nM) (Figure 2d), indicating that AMPA-mediated signaling drives mTOR-dependent dendrite outgrowth in mesencephalic DA neurons. The involvement of AMPAR in ketamine-induced structural plasticity was likewise studied using NBQX (0.01-10 μM) as well as the AMPAR negative allosteric modulator (NAM) GYKI 52466 (0.01-10 μM). Both compounds dose-dependently blocked the effects of ketamine (Figure 2e). In a series of acute phosphorylation studies using western blot we showed that pretreatment with NQBX (10 μM) blocked the peak of p-p70S6K observed 5 min after ketamine exposure, further supporting AMPAR-mediated structural plasticity *via* activation of the mTOR pathway (Figure 2f).

We further studied the role of NMDAR in the downstream activation of AMPAR using the selective NMDAR/NR2B antagonist Ro 25-6981. Ro 25-6981 produced a concentration-dependent (0.01-1 μM) increase of structural plasticity, with a minimal effective concentration (statistically significant action) of 0.1 μM (Supplementary Figure S3c). Pretreatments with NBQX or GYKI 52466 completely prevent the effects of 1 μM Ro 25-6981 (Figure 2g), confirming the role of AMPAR activation in structural plasticity produced by NMDAR/NR2B antagonism (Figure 2h).

*BDNF-TrkB signaling is involved in structural plasticity induced by ketamine.*

Activation of BDNF-TrkB signaling upstream to mTOR pathway is considered a critical step in mediating the antidepressant effects of ketamine and structural plasticity in frontocortical-hippocampal circuits.[5,13,16] Hence, we assessed if BDNF-TrkB signaling has a similar role in DA neurons. We first examined the influence on ketamine-induced structural plasticity of either an anti-BDNF blocking antibody

(α–BDNF) or a TrkB-Fc Chimera[13] (a cell membrane-impermeable BDNF scavenger). They were applied to neuronal cultures 20 min before a 60-min exposure to ketamine. Both agents fully blocked the effects of ketamine, while they were inactive when applied alone (Figure 3a). The effects of ketamine were also blocked by targeting intracellular BDNF-TrkB signaling using the TrkB phosphorylation inhibitor K252a, the TrkB-dependent Src phosphorylation inhibitor PP2 and the MEK-phosphorylation inhibitor PD98059 (Figure 3b). Pretreatment with α–BDNF or TrkB-Fc Chimera were also effective in blocking structural plasticity induced by CX614 (Figure 3c) in DA neurons, in line with previous observations in rodent telencephalic neurons.[13] These data support a role of AMPAR-dependent BDNF/TrkB signaling in the structural plasticity induced by ketamine in mesencephalic DA neurons.

*Role of dopamine D3 receptors in ketamine-induced structural plasticity.*

We previously showed that DA agonists produce structural plasticity in cultured mouse DA neurons *via* D3R-dependent activation of ERK and PI3K-mTOR pathways.[38,39,40] This signaling partially overlaps with intracellular signaling so far described for ketamine (Figure 4h), suggesting a possible interaction. Here we studied the contribution of D3R activation or blockade to ketamine-induced structural plasticity. Pretreatment with the selective D3R antagonists SB277011-A[55] and S33084[56] blocked ketamine-induced structural plasticity and this effect was specific inasmuch as it was not observed with the selective D1 receptor (D1R) antagonist SCH23390[57] (Figure 4a). The lack of effect of SCH23390 was expected since D1Rs are not expressed in mesencephalic DA neurons.[57] Pretreatment with SB277011-A also inhibited the effect of CX614 (Figure 4b), suggesting a permissive role of DA D3R in AMPAR-mediated structural plasticity.

Using western blot, we studied the contribution of D3R to the PI3K-mTOR pathway activation induced by ketamine. Ketamine effects on p-p70S6K were antagonized by pretreatment with SB277011-A (Figure 1c). Parallel immunofluorescence experiments showed that the effect of ketamine on p-p70S6K was antagonized by pretreatment with SB277011-A in $TH^+$ neurons (Figure 4d and e). Sampling of $TH^-$ neurons showed that SB277011-A also partially blocked the ketamine-induced p-p70S6K increase. These findings were confirmed in mesencephalic DA neurons obtained from D3KO mice and wild-type mice: ketamine, the D3-preferential agonist 7-OH-DPAT and BDNF induced structural plasticity in wild-type DA neurons, whereas BDNF was the only agent to be effective in D3KO DA neuronal cultures (Figure 3f). In acute phosphorylation experiments, ketamine and 7-OH-DPAT rapidly increased p-p70S6K in DA neurons from cultures of wild-type mice, but not of D3KO mice (Figure 4g). These data support the involvement of D3R signaling in PI3K-mTOR-mediated effects of ketamine on structural plasticity of DA neurons.

*Ketamine induces structural plasticity in human iPSCs derived DA neurons.*

We studied the effects of ketamine on human DA neurons differentiated from iPSCs. After 80 days *in vitro*, the cultures contained 31%±4% $TH^+$ neurons, 22%±5% $GAD67^+$ neurons and 29%±8% $VGLUT2^+$ neurons co-stained with anti-MAP2 antibody, these $MAP2^+$ neurons constituting 80-90% of the total number of cells in the culture (Supplementary Figure S4c and d). $TH^+$ neurons consistently expressed DAT indicating a mature DA phenotype (Supplementary Figure S4b). Similarly to mouse mesencephalic cultures, $TH^+$ neurons expressed NMDAR/NR2B subunits and AMPAR subunits (Supplementary Figure S4e, g and h). GABAergic neurons also expressed the NMDAR/NR2B subunit (Supplementary Figure S4f).

Exposure to ketamine (0.01-1 μM) for 60 min elicited a concentration-dependent increase in structural plasticity measured after 72 hrs, similar to that observed in mouse cultures (Supplementary Figure S5a). Further, the NMDAR NR2B antagonist Ro 25-6981, when tested at the same concentration used in mouse cultures, likewise promoted plasticity (Figure 5a and b). Structural plasticity elicited by ketamine was blocked by pretreatment with NBQX or GYKI 52466 (Figure 5b), supporting the involvement of AMPAR. The critical role of AMPAR activation was confirmed using CX614 that revealed a concentration-dependent induction of structural plasticity (Supplementary Figure S5b and c). The effects of CX614 were blocked by pretreatment with NBQX and rapamycin (Supplementary Figure S5d). The involvement of BDNF-TrkB signaling was underpinned by using the same experimental approach as in the mouse studies, i.e., an α–BDNF antibody and a TrkB-Fc Chimera (Figure 5c) as well as the TrkB/MEK inhibitors K252a, PP2 and PD98059 (Figure 5d). All blocked the increase in plasticity evoked by ketamine in human DA neurons. A role of D3R in mediating the effects of ketamine on structural plasticity in human DA neurons was demonstrated using the D3R antagonists SB277011-A and S33084 (Figure 5e). We also established the involvement of the PI3K-mTOR pathway using LY294002 and rapamycin (Figure 5f). A pattern similar to what observed in mouse DA neurons was also observed in the acute phosphorylation experiments. Using western blot, ketamine induced a rapid increase of p-p70S6K levels with a peak 5 min after exposure, an effect that lasted 60 min (Figure 5g). Parallel immunofluorescence experiments (Figure 5h) showed that ketamine significantly increased the basal levels of p-p70S6K in $TH^+$ neurons, a positive effect was likewise seen in $TH^-$ neurons (Figure 5h and i).

*(2R,6R)-HNK induces structural plasticity in mouse mesencephalic and human iPSCs derived DA neurons.*

Recent *in vivo* studies have shown that the ketamine metabolite (2R,6R)-HNK produces rapid and sustained antidepressant effects in rodents.[51] Since in human (2R,6R)-HNK is the major metabolite of ketamine and its levels remain elevated for several hours in sub-micromolar range,[58] we studied the effects of 0.1-0.5 μM (2R,6R)-HNK on structural plasticity at the exposure time of 60 min and 6 hrs. At the concentration of 0.5 μM, (2R,6R)-HNK produced a significant increase of structural plasticity in both mouse mesencephalic and human iPSC-derived DA neurons (Table 1 and 2), showing trends for increases at 0.1 μM. All increases were visible at both time points, showing stronger effects at 6 hrs after dosing.

**Discussion**

Using *in vitro* models of primary DA neurons from mouse embryo mesencephalon and human iPSC-derived DA neurons, the present study demonstrates for the first time that transient exposure to ketamine dose-dependently promotes structural plasticity as determined by enhanced dendritic outgrowth and increased soma size. The molecular mechanisms underpinning the influence of ketamine on structural plasticity of mouse and human DA neurons involved both BDNF and mTOR signaling and the upstream activation of AMPAR. Their implication mirrors their role in the control of dendritic remodeling and structural plasticity in frontocortical and hippocampal neurons.[15,16,17,59] In addition, we identify a novel role for dopamine D3R-dependent signaling in the actions of ketamine.

The effects of ketamine on structural plasticity were paralleled by the selective NMDAR/NR2B antagonist Ro 25-6981,[49] a compound that shares with ketamine antidepressant effects and frontocortical/hippocampal circuit remodelling in mice.[8,11,15] Ro 25-6981, which was shown to be more potent than ketamine on [3H]MK-801 binding both *in vitro* (about 20-fold) and *in vivo* (about 4-fold),[60] correspondingly promoted structural plasticity in DA neurons at a concentration 10-fold lower than ketamine (0.1-1 μM). We also observed that the ketamine metabolite (2R,6R)-HNK induced structural plasticity in both mice and human DA neurons at sub-micromolar concentrations. Recent findings indicate that (2R,6R)-HNK has antidepressant-like properties in mice.[51] It was proposed that some of the long-lasting pharmacological effects of ketamine are mediated by its metabolites *in vivo*.[51,58,61] In our *in vitro* preparations we did not find metabolites, supporting ketamine direct effect. In the present study the effective concentrations of both ketamine (1-10 μM) and (2R,6R)-HNK (0.5 μM) for promoting neuroplasticity were 5-10 times lower than

those observed by electrophysiology on hippocampal neurons *in vitro*,[61] a difference probably related to the different neural phenotype tested (i.e., mesencephalic DA vs. hippocampal), the different exposure time (i.e., sec/min for electrophysiology vs. hours for plasticity) and the fact that electrophysiological studies were undertaken in media free of $Mg^{++}$ that behaves as an open channel blocker at NMDAR. All these factors may account for the need for higher concentrations of ketamine in electrophysiological works. Interestingly, human pharmacokinetic studies indicated that the peak plasma levels of ketamine and (2R,6R)-HNK following therapeutic sub-anaesthetic infusion of ketamine in patients with mood disorders[58] are in the same order of magnitude as concentrations effective herein for inducing structural plasticity in DA neurons.

Pharmacological inhibitors of the mTOR intracellular pathway profoundly affected ketamine-induced structural plasticity in both mice and human DA neurons. One of these inhibitors, rapamycin, was previously shown to block ketamine-dependent dendritic spine remodelling in frontocortical neurons.[15] Indeed, mTOR pathway was rapidly activated by ketamine, as indicated by the increased p-p70S6K levels in both mouse and human DA neurons, peaking at 5 min and remaining elevated up to 60 min. The activation of mTOR pathway of both DA and non-DA neurons was expected since NMDAR are expressed also in GABAergic and glutamatergic neurons.[8,10]

The parallels in molecular substrates engaged by ketamine in neurons of frontocortical/hippocampal circuits and mesencephalic DA system do not stop here: in both systems the promotion of structural plasticity induced by ketamine (and Ro 25-6981) was dependent upon functionally-intact AMPAR,[11,15,17,62] as showed by the blocking effects of AMPAR antagonists or direct activation using the AMPAR PAM

CX614 that reproduced the actions of ketamine in DA neurons. Converging findings indicate a role of an enhanced AMPAR transmission in mediating the behavioural antidepressant properties of ketamine and AMPAR PAMs in rodents[6,8,11,12] and humans.[63] Intriguingly, enhanced AMPAR neurotransmission was observed in DA neurons of transgenic mice upon selective inactivation of NMDAR/NR1 achieved by crossing floxed NR1 mice with mice carrying Cre recombinase driven by DAT promoter.[64] The findings of our cellular models are in keeping with this literature contributing to the interpretation of the central role of AMPAR.

BDNF-TrkB signaling has been extensively studied as a potential substrate for the influence of antidepressants on functional[65] and structural plasticity.[24,26,66] Increased BDNF synthesis and release in rodent frontocortical/hippocampal circuits were observed after acute exposure to either ketamine[5,14,17] or AMPAR PAMs.[13,54,62] Further, BDNF-TrkB signaling is critical for recruiting the molecular machinery involved in structural dendritic outgrowth and remodelling.[13,16,59] In the present work, using the same blocking agents as those used to dissect out mechanisms of AMPAR-dependent BDNF-TrkB signaling in hippocampal neurons,[13] a BDNF-TrkB cascade was shown to be necessary for mediating the influence of both ketamine and the AMPAR PAM CX614 on structural plasticity in DA neurons. Since low BDNF expression in the VTA and dysfunctional DA neurotransmission are core features of anhedonia,[29,67] ketamine may conceivably exerts its sustained antidepressant effects by activating BDNF/TrkB signaling to enhance structural (and functional[44]) plasticity of DA system. Supporting evidence in rodents includes the antidepressant-like effects produced by exogenous BDNF infusions in VTA[68] and increases of BDNF expression in both hippocampus and VTA upon chronic treatment with fluoxetine.[69] However, while a certain degree of BDNF-TrkB signaling could be beneficial for the stress-

coping response,[67] excessive and prolonged enhancement of BDNF-TrkB signaling in the VTA *via* transgenic overexpression produces an aversive motivational state and increased vulnerability to stress,[28,70] suggesting the need of calibrated regulation.

The present study provides novel and robust evidence that D3Rs are involved in the effects of ketamine on DA neurons. In a series of recent studies, we[38,39,40] and others[71,72] showed that D3R activation leads to structural plasticity in DA neurons, an effect transduced by intracellular ERK and PI3K-dependent-mTOR signaling. Using selective D3R antagonists and D3KO mice, D3R-mediated neurotransmission was shown to be necessary for the rapid activation of mTOR signaling and the delayed enhanced dendritic outgrowth elicited by ketamine and CX614. Since mTOR phosphorylation is also triggered by BDNF-induced TrkB activation,[16] we propose a convergence between the D3R and BDNF-TrkB pathways to promote structural plasticity. In line with this interpretation, in a study on rats with nigrostriatal lesions, nanovector-mediated BDNF overexpression enhanced the effects of the D3R preferential DA agonist 7-OH-DPAT on the structural plasticity of mesencephalic DA neurons.[73] By analogy to mouse mesencephalic cultures, functionally intact D3R signaling was also necessary in human iPSC-derived DA neurons for the actions of ketamine. Intriguingly, human PET studies have shown that almost all binding sites in the ventral mesencephalon are of the D3R type,[74] supporting the clinical relevance of our findings also in light of the antidepressant effects observed with the D3R-preferential DA agonist pramipexole.[33,34]

In conclusion, this work explored cellular mechanisms underpinning the influence of ketamine upon structural plasticity in DA neurons using an *in vitro* paradigm based on the parallel assessment of mice primary neural cultures *and* human iPSC-derived neurons. The remarkable similarities in the influence of ketamine across

these two cellular paradigms mutually support the role of AMPAR, mTOR, BDNF and D3R signaling in the actions of ketamine. From a translational perspective, the comparable data seen in human iPSC-derived and mouse DA neurons strongly underscores the relevance of the present work to the pharmacological actions of ketamine in humans. However, in addition to its antidepressant properties, like other channel blockers of NMDA receptors, ketamine exerts psychoactive effects and can even provoke psychosis at high doses[2]. Accordingly, structural remodelling of mesencephalic DA neurons might be involved in actions of ketamine *other* than the alleviation of depressed mood. Further cellular and *in vivo* work will be required to clarify the precise functional significance of the present findings.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgments**

The authors thank Cristina Mora for PCR analysis, Florent Meunier and Marie Hideux for HPLC and mass spectrometry of ketamine in culture media, Marzia Di Chio for technical assistance, Verdon Taylor for critical comments on the manuscript and figures.

Research was supported by: Grant from ex 60%, University of Brescia to G.C., and by MIUR ex-60% research funds to C.C.

**References**


1. Sinner B, Graf BM. Ketamine. *Handb Exp Pharmacol*. 2008; **182**: 313-333.
2. Krystal JH, Karper LP, Seibyl JP, Freeman GK, Delaney R, Bremner JD et al. Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychotomimetic, perceptual, cognitive, and neuroendocrine responses. *Arch Gen Psychiatry* 1994; **51**: 199-214.
3. Zarate CA, Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA et al. A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch Gen Psychiatry* 2006; **63**: 856-864.
4. Coyle CM, Laws KR. The use of ketamine as an antidepressant: a systematic review and meta-analysis. *Hum Psychopharmacol* 2015; **30**: 152-163.
5. Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF et al. NMDA receptor blockade at rest triggers rapid behavioral antidepressant responses. *Nature* 2011; **475**: 91-95.
6. Browne CA, Lucki I. Antidepressant effects of ketamine: mechanisms underlying fast-acting novel antidepressants. *Front Pharmacol* 2013; **4**: 161.
7. Yilmaz A, Schulz D, Aksoy A, Canbeyli R. Prolonged effect of an anesthetic dose of ketamine on behavioral despair. *Pharmacol Biochem Behav* 2002; **71**: 341-344.
8. Krystal JH, Sanacora G, Duman RS. Rapid-acting glutamatergic antidepressants: the path to ketamine and beyond. *Biol Psychiatry* 2013; **73**: 1133-1141.
9. Homayoun H, Moghaddam B. NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J Neurosci* 2007; **27:** 11496-11500.

10. Miller OH, Moran JT, Hall BJ. Two cellular hypotheses explaining the initiation of ketamine's antidepressant actions: Direct inhibition and disinhibition. *Neuropharmacology* 2016; **100**: 17-26.
11. Maeng S, Zarate CA Jr, Du J, Schloesser R J, Mc Cammon J, Chen G et al. Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5 methylisoxazole-4-propionic acid receptors. *Biol Psychiatry* 2008; **63**: 349-352.
12. Koike H, Iijima M, Chaki S. Involvement of AMPA receptor in both the rapid and sustained antidepressant-like effects of ketamine in animal models of depression. *Behav Brain Res* 2011; **224**: 107-111.
13. Jourdi H, Hsu YT, Zhou M, Qin Q, Bi X, Baudry M. Positive AMPA receptor modulation rapidly stimulates BDNF release and increases dendritic mRNA translation. *J Neurosci* 2009; **29**: 8688-8697.
14. Lepack AE, Fuchikami M, Dwyer JM, Banasr M, Duman RS. BDNF release is required for the behavioral actions of ketamine. *Int J Neuropsychopharm* 2014; **18**: 1-6.
15. Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M et al. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* 2010; **329**: 959-964.
16. Takei N, Inamura N, Kawamura M, Namba H, Hara K, Yonezawa K et al. Brain-Derived Neurotrophic Factor induces Mammalian Target of Rapamycin dependent local activation of translation machinery and protein synthesis in neuronal dendrites. *J Neurosci* 2004; **24**: 9760-9769.
17. Zhou W, Wang N, Yang C, Li XM, Zhou ZQ, Yang JJ. Ketamine-induced antidepressant effects are associated with AMPA receptors-mediated

upregulation of mTOR and BDNF in rat hippocampus and prefrontal cortex. *European Psychiatry* 2014; **29**: 419-423.

18. Drevets WC, Price JL, Furey ML. Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Struct Funct* 2008; **213**: 93-118.
19. Sanacora G, Treccani G, Popoli M. Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacol* 2012; **62**: 63-77.
20. Jernigan CS, Goswami DB, Austin MC, Iyo AH, Chandran A, Stockmeier CA et al. The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry* 2011; **35**: 1774-1779.
21. Christoffel DJ, Golden SA, Russo SJ. Structural and synaptic plasticity in stress-related disorders. *Rev Neurosci* 2011; **22**: 535-549.
22. Polman JA, Hunter RG, Speksnijder N, van den Oever JM, Korobko OB, McEwen BS et al. Glucocorticoids modulate the mTOR pathway in the hippocampus: differential effects depending on stress history. *Endocrinology* 2012; **153**: 4317-4327.
23. Chen F, Madsen TM, Wegener G, Nyengaard JR. Repeated electroconvulsive seizures increase the total number of synapses in adult male rat hippocampus. *Eur Neuropsychopharmacol* 2009; **19**: 329-338.
24. Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA et al. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry* 2009; **14**: 764-777.

25. Dukart J, Regen F, Kherif F, Colla M, Bajbouj M, Heuser I et al. Electroconvulsive therapy-induced brain plasticity determines therapeutic outcome in mood disorders. *Proc Natl Acad Sci USA* 2014; **111**: 1156-1161.
26. Castrén E, Rantamäki T. The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. *Dev Neurobiol* 2010; **70**: 289-297.
27. Schmidt U, Beyer C, Oestreicher AB, Reisert I, Schilling K, Pilgrim C. Activation of dopaminergic D1 receptor promotes morphogenesis of developing striatal neurons. *Neuroscience* 1996; **74:** 453-460.
28. Nestler EJ, Carlezon WA. The mesolimbic dopamine reward circuit in depression. *Jr Biol Psychiatry* 2006; **59**: 1151-1159.
29. Der-Avakian A, Mazei-Robison MS, Kesby JP, Nestler EJ, Markou A. Enduring deficits in brain reward function after chronic social defeat in rats: susceptibility, resilience, and antidepressant response. *Biol Psychiatry* 2014; **76**: 542-549.
30. Meyer JH, Krüger S, Wilson AA, Christensen BK, Goulding VS, Schaffer A et al. Lower dopamine transporter binding potential in striatum during depression. *Neuroreport.* 2001; **12**: 4121-4125.
31. Vassout A, Bruinink A, Krauss J, Waldmeier P, Bischoff S. Regulation of dopamine receptors by bupropion: comparison with antidepressants and CNS stimulants. *J Recept Res.* 1993; **13**: 341-354.
32. Leggio GM, Salomone S, Bucolo C, Platania C, Micale V, Caraci F et al. Dopamine D(3) receptor as a new pharmacological target for the treatment of depression. *Eur J Pharmacol* 2013; **719**: 25-33.

33. Breuer ME, Groenink L, Oosting RS, Buerger E, Korte M, Ferger B et al. Antidepressant effects of pramipexole, a dopamine D3/D2 receptor agonist, and 7-OH-DPAT, a dopamine D3 receptor agonist, in olfactory bulbectomized rats. *Eur J Pharmacol* 2009; **616**: 134-140.
34. Barone P, Poewe W, Albrecht S, Debieuvre C, Massey D, Rascol O et al. Pramipexole for the treatment of depressive symptoms in patients with Parkinson's disease: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol* 2010; **9**: 573-580.
35. Diaz J, Ridray S, Mignon V, Griffon N, Schwartz JC, Sokoloff P. Selective expression of dopamine D3 receptor mRNA in proliferative zones during embryonic development of the rat brain. *J Neurosci.* 1997; **17**: 4282-4292.
36. Gurevich EV, Joyce JN. Distribution of dopamine D3 receptor expressing neurons in the human forebrain: comparison with D2 receptor expressing neurons. *Neuropsychopharmacology* 1999; **20**: 60-80.
37. Sun J, Xu J, Cairns NJ, Perlmutter JS, Mach RH. Dopamine D1, D2, D3 receptors, vesicular monoamine transporter type-2 (VMAT2) and dopamine transporter (DAT) densities in aged human brain. *PLoS One* 2012; **7**(11):e49483.
38. Collo G, Zanetti S, Missale C, Spano PF. Dopamine D3 receptor-preferring agonists increase dendrite arborization of mesencephalic dopaminergic neurons via extracellular signal-regulated kinase phosphorylation. *Eur J Neurosci* 2008; **28**: 1231-1240.
39. Collo G, Bono F, Cavalleri L, Plebani L, Merlo Pich E, Millan MJ et al. Pre-synaptic dopamine D3 receptor mediates cocaine-induced structural plasticity

in mesencephalic dopaminergic neurons via ERK and Akt pathways. *J Neurochem* 2012; **120**: 765-778.

40. Collo G, Bono F, Cavalleri L, Plebani L, Mitola S, Merlo Pich E et al. Nicotine-induced structural plasticity in mesencephalic dopaminergic neurons is mediated by dopamine D3 receptors and Akt-mTORC1 signaling. *Mol Pharm* 2013; **83**: 1176-1189.
41. Lindefors N, Barati S, O'Connor WT. Differential effects of single and repeated ketamine administration on dopamine, serotonin and GABA transmission in rat medial prefrontal cortex. *Brain Res* 1997; **759**: 205-212.
42. Kegeles LS, Abi-Dargham A, Zea-Ponce Y, Rodenhiser-Hill J, Mann JJ, Van Heertum RL et al. Modulation of amphetamine-induced striatal dopamine release by ketamine in humans: implications for schizophrenia. *Biol Psychiatry* 2000; **48**: 627-640.
43. Can A, Zanos P, Moaddel R, Kang HJ, Dossou KS, Wainer IW et al. Effects of ketamine and ketamine metabolites on evoked striatal dopamine release, dopamine receptors, and monoamine transporters. *J Pharmacol Exp Ther.* 2016; **359**: 159-170.
44. Belujon P, Grace AA. Restoring mood balance in depression: ketamine reverses deficit in dopamine-dependent synaptic plasticity. *Biol Psychiatry* 2014; **76**: 927-936.
45. Devine MJ, Ryten M, Vodicka P, Thomson AJ, Burdon T, Houlden H et al. Parkinson's disease induced pluripotent stem cells with triplication of the α-synuclein locus. *Nat Commun* 2011; **2**: 440.

46. Kriks S, Shim JW, Piao J, Ganat YM, Wakeman DR, Xie Z et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 2011; **480**: 547-551.

47. Avior Y, Sagi I, Benvenisty N. Pluripotent stem cells in disease modelling and drug discovery. *Nat Rev Mol Cell Biol.* 2016; **17**: 170-182.

48. Accili D, Fishburn CS, Drago J, Steiner H, Lachowicz JE, Park B-H et al. A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. *Proc Natl Acad Sci USA* 1996; **93**: 1945-1949.

49. Fischer G, Mutel V, Trube G, Malherbe P, Kew JN, Mohacsi E et al. Ro 25-6981, a highly potent and selective blocker of N-methyl-D-aspartate receptors containing the NR2B subunit. Characterization in vitro. *J Pharmacol Exp Ther* 1997; **283**: 1285-1292.

50. Arai AC, Kessler M, Rogers G, Lynch G. Effects of the potent ampakine CX614 on hippocampal and recombinant AMPA receptors: interactions with cyclothiazide and GYKI52466. *Mol Pharmacol* 2000; **58**: 802-813.

51. Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI et al. NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature* 2016; **533**: 481-486.

52. Chen L-W, Weib L-C, Langa B, Jua G, Chanc YS. Differential expression of AMPA receptor subunits in dopamine neurons of the rat brain: a double immunocytochemical study. *Neuroscience* 2001; **106**: 149-160.

53. Chen W, Prithviraj R, Mahnke AH, McGloin KE, Tan JW, Gooch AK et al. AMPA glutamate receptor subunits 1 and 2 regulate dendrite complexity and spine motility in neurons of the developing neocortex. *Neuroscience* 2009; **159**: 172-182.

54. Lauterborn JC, Palmer LC, Jia Y, Pham DT, Hou B, Wang W et al. Chronic ampakine treatments stimulate dendritic growth and promote learning in middle-aged rats. *J Neurosci* 2016; **36**: 1636-1646.

55. Reavill C, Taylor SG, Wood MD, Ashmeade T, Austin NE, Avenell KY et al. Pharmacological actions of a novel, high-affinity, and selective human dopamine D(3) receptor antagonist, SB277011-A. *J Pharmacol Exp Ther* 2000; **294**: 1154-1165.

56. Millan MJ, Gobert A, Newman-Tancredi A, Lejeune F, Cussac D, Rivet JM et al. S33084, a novel, potent, selective, and competitive antagonist at dopamine D(3)-receptors: I. Receptorial, electrophysiological and neurochemical profile compared with GR218,231 and L741,626. *J Pharmacol Exp Ther* 2000; **293**: 1048-1062.

57. Meador-Woodruff JH, Mansour A, Healy DJ, Kuehn R, Zhou QY, Bunzow JR et al. Comparison of the distributions of D1 and D2 dopamine receptor mRNAs in rat brain. *Neuropsychopharmacol* 1991; **5**: 231-242.

58. Zhao X, Venkata SL, Moaddel R, Luckenbaugh DA, Brutsche NE, Ibrahim L et al. Simultaneous population pharmacokinetic modelling of ketamine and three major metabolites in patients with treatment-resistant bipolar depression. *Br J Clin Pharmacol* 2012; **74**: 304-314.

59. Kumar V, Zhang M-X, Swank MW, Kunz J, Wu G-Y. Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. *J Neurosci* 2005; **25**: 11288-11299.

60. Murray F, Kennedy J, Hutson PH, Elliot J, Huscroft I, Mohnen K et al. Modulation of [3H]MK-801 binding to NMDA receptors in vivo and in vitro. *Eur J Pharmacol.* 2000; **397**: 263-270.

61. Suzuki K, Nosyreva E, Hunt KW, Kavalali ET, Monteggia LM. Effects of a ketamine metabolite on synaptic NMDAR function. *Nature* 2017; **546**: E1-E3.
62. Lauterborn JC, Truong GS, Baudry M, Bi X, Lynch G, Gall CM. Chronic elevation of brain-derived neurotrophic factor by ampakines. *J Pharmacol Exp Ther* 2003; **307**: 297-305.
63. Nations KR, Dogterom P, Bursi R, Schipper J, Greenwald S, Zraket D et al. Examination of Org 26576, an AMPA receptor positive allosteric modulator, in patients diagnosed with major depressive disorder: an exploratory, randomized, double-blind, placebo-controlled trial. *J Psychopharmacol* 2012; **26**: 1525-1539.
64. Zweifel LS, Argilli E, Bonci A, Palmiter RD. Role of NMDA receptors in dopamine neurons for plasticity and addictive behaviors. *Neuron* 2008; **59**: 486-496.
65. Alme MN, Wibrand K, Dagestad G, Bramham CR. Chronic fluoxetine treatment induces brain region-specific upregulation of genes associated with BDNF induced long-term potentiation. *Neural Plast.* 2007; **2007**: 26496.
66. Björkholm C, Monteggia LM. BDNF - a key transducer of antidepressant effects. *Neuropharmacology* 2016; **102**:72-79.
67. Miczek KA, Nikulina EM, Shimamoto A, Covington HE 3rd. Escalated or suppressed cocaine reward, tegmental BDNF, and accumbal dopamine caused by episodic versus continuous social stress in rats. *J Neurosci*. 2011; **31**: 9848-9857.
68. Siuciak JA, Lewis DR, Wiegand SJ, Lindsay RM. Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). *Pharmacol Biochem Behav.* 1997; **56**: 131-137.

69. Molteni R, Calabrese F, Bedogni F, Tongiorgi E, Fumagalli F, Racagni G et al. Chronic treatment with fluoxetine up-regulates cellular BDNF mRNA expression in rat dopaminergic regions. *Int J Neuropsychopharmacol*. 2006; **9**: 307-317.

70. Wook Koo J, Labonté B, Engmann O, Calipari ES, Juarez B, Lorsch Z et al. Essential role of mesolimbic Brain-Derived Neurotrophic Factor in Chronic Social Stress-induced depressive behaviors. *Biol Psychiatry* 2016; **80**: 469-478.

71. Van Kampen JM, Eckman CB. Dopamine D3 receptor agonist delivery to a model of Parkinson's disase restores the nigrostriatal pathway and improves locomotor behavior. *J Neurosci* 2006; **26**: 7272-7280.

72. Mueller D, Chapman CA, Stewart J. Amphetamine induces dendritic growth in ventral tegmental area dopaminergic neurons in vivo via basic fibroblast grow factor. *Neuroscience* 2006; **137**: 727-735.

73. Razgado-Hernandez LF, Espadas-Alvarez AJ, Reyna-Velazquez P, Sierra-Sanchez A, Anaya-Martinez V, Jimenez-Estrada I et al. The transfection of BDNF to dopamine neurons potentiates the effect of dopamine D3 receptor agonist recovering the striatal innervation, dendritic spines and motor behavior in an aged rat model of Parkinson's disease. *PLoS One* 2015; **10**:e0117391.

74. Searle G, Beaver JD, Comley RA, Bani M, Tziortzi A, Slifstein M et al. Imaging dopamine D3 receptors in the human brain with positron emission tomography, [11C]PHNO, and a selective D3 receptor antagonist. *Biol Psychiatry* 2010; **68**: 392-399.

**Figure legends**

**Figure 1. Ketamine-induced structural plasticity in mouse mesencephalic dopaminergic (DA) neurons depends upon activation of the mTOR pathway**. (**a**) Representative photomicrographs of $TH^+$ neurons at 72 hrs after initial transient exposure (60 min) to 1 μM ketamine (right panel) or vehicle (left panel). Scale bar: 50 μm. (**b**) Morphometric assessment of $TH^+$ neurons performed at 72 hrs after an initial transient exposure (60 min) to various doses of ketamine (0.001-10 μM) or vehicle. Three structural plasticity parameters were measured: maximal dendrite length [One-way ANOVA $F(5,174) = 6.29$, $P<0.0001$], primary dendrite number [Kruskal-Wallis $(5,300) = 16.3$, $P<0.01$] and soma area [One-way ANOVA $F(5,234) = 5.28$, $P=0.0001$]. 0 = Vehicle. (**c**) Inhibition of ketamine-induced structural plasticity following pretreatment (20 min) with either the PI3K inhibitor LY294002 (10 μM) or the mTORC1 inhibitor rapamycin (20 nM), measured 72 hrs after initial transient exposure (60 min) to 1 μM ketamine: maximal dendrite length [Two-way ANOVA interaction: $F(2,174) = 10$, $P<0.0001$; treatment factor: $F(1,174) = 8.7$, $P<0.005$; inhibition factor: $F(2,174) = 0.88$, ns], primary dendrite number [Friedman $(5,300) = 15.1$, $P<0.001$] and soma area [Two-way ANOVA interaction: $F(2,234) = 6.2$, $P<0.005$; treatment factor: $F(1,234) = 13.6$, $P<0.0005$; inhibition factor: $F(2,234) = 4.4$, $P<0.05$]. (**d**) Effects of transient exposure (2-60 min) to 1 μM ketamine on phosphorylated p70S6K (p-p70S6K) levels measured with western blot and analyzed by densitometry [One-way ANOVA $F(5,24) = 4.97$, $P<0.01$]; p-p70S6K levels were normalised to the corresponding p70S6K, TH and tubulin levels. The densitometric values are represented as percentage of vehicle values. (**e**) Blockade of increase of p-p70S6K levels induced by 1 μM ketamine after pretreatment with LY294002 (10 μM)

analyzed by densitometry on normalized western blots [Two-way ANOVA interaction: $F(1,12) = 4.8$, $P<0.05$; treatment factor: $F(1,12) = 10.5$, $P<0.01$; inhibition factor: $F(1,12) = 14.0$, $P<0.005$]. **(f)** Representative photomicrographs of $TH^+$ neurons (red) expressing p-p70S6K (green) after vehicle**,** 1 μM ketamine**,** pretreament with LY294002 (10 μM) followed by 1 μM ketamine or LY294002 (10 μM) alone. (**g**) Semiquantitative image analysis of p-p70S6K fluorescence intensity. In $TH^+$ neurons, the significant increase of p-p70S6K is attenuated by LY294002 pre-treatment [Two-way ANOVA interaction: $F(1,116) = 33.0$, $P<0.0001$; treatment factor: $F(1,116) = 11.5$, $P<0.001$; inhibition factor: $F(1,116) = 38.5$, $P<0.0001$]; in a sample of $TH^-$ neurons a similar effect was observed [Two-way ANOVA interaction: $F(1,243) = 15.1$, $P<0.0001$; treatment factor: $F(1,243) = 27.4$, $P<0.0001$; inhibition factor: $F(1,243) = 11.4$, $P<0.001$]. Experiments of pharmacologic antagonism on p-p70S6K levels were performed at 5 min after exposure to ketamine to obtain the maximal dynamic range. Cell nuclei were stained with DAPI. Scale bar: 50 μm. In all panels, data are expressed as mean ± S.E.M. (***$P<0.001$, **$P<0.01$, *$P<0.5$ vs. vehicle; $^{ooo}P<0.001$, $^{oo}P<0.01$, $^{o}P<0.05$ vs. ketamine, *post-hoc* Bonferroni's or Dunn's test). V = vehicle; K = ketamine; LY = LY294002; R = rapamycin.

**Figure 2. Ketamine-induced structural plasticity is mediated *via* AMPAR activation.** (**a** and **b**) Representative photomicrographs showing co-distribution of $TH^+$ (red) and $GluR1^+$ (green) (**a**) or $GluR2^+$ (green) (**b**), respectively. Cell nuclei were stained with DAPI. Scale bar: 20 μm. (**c**) Semiquantitative image analysis of $GluR1^+$ and $GluR2^+$ fluorescence intensity in soma vs. dendrites of $TH^+$ neurons [Two-way ANOVA interaction: $F(1,116) = 40.8$, $P<0.0001$; GLUR factor: $F(1,116) = 0.01$, ns; soma/dendrites factor: $F(1,116) = 0.9$, ns]. (**d**) Inhibition of the effects of the

AMPAR positive allosteric modulator CX614 (10 μM) on structural plasticity following pretreatment (20 min) with either the AMPAR antagonist NBQX (10 μM) or rapamycin (20 nM) measured after 72 hrs: maximal dendrite length [Two-way ANOVA interaction: $F(2,174) = 7.6$, $P<0.001$; treatment factor: $F(1,174) = 12.2$, $P<0.001$; inhibition factor: $F(2,174) = 3.8$, $P<0.05$], primary dendrite number [Friedman $(5,300) = 12.2$, $P<0.05$] and soma area [Two-way ANOVA interaction: $F(2,234) = 8.3$, $P<0.0005$; treatment factor: $F(1,234) = 1.5$, ns; inhibition factor: $F(2,234) = 7.9$, $P<0.0005$]. (**e**) Concentration-dependent inhibition of 60 min ketamine-induced structural plasticity following pretreatment with either NBQX (0.01-10 μM) or GYKI52466 (0.01-10 μM) on maximal dendrite length [NBQX: One-way ANOVA $F(5,174) = 7.11$, $P<0.0001$ and GYKI52466: One-way ANOVA $F(5, 174) = 5.57$, $P<0.0001$], primary dendrite number [NBQX: Kruskal-Wallis $(5,300) = 15.3$, $P<0.01$ and GYKI52466: Kruskal-Wallis $(5,300) = 15.4$, $P<0.01$] and soma area [NBQX: One-way ANOVA $F(5,234) = 3.92$, $P<0.001$ and GYKI52466: One-way ANOVA $F(5,234) = 5.26$, $P<0.0001$]. Data are expressed as mean of % maximal ketamine effect; ANOVA was performed on the raw data. (**f**) Blockade of increase of p-p70S6K levels induced by 1 μM ketamine after pretreatment with NBQX (10 μM) measured 5 min after exposure and analyzed by densitometry on normalized western blots [Two-way ANOVA interaction: $F(1,19) = 11.3$, $P<0.005$; treatment factor: $F(1,19) = 2.4$, ns; inhibition factor: $F(1,19) = 6.4$, $P<0.02$]. (**g**) Inhibition of structural plasticity induced by the selective NMDAR/NR2B antagonist Ro 25-6981 (1 μM) following pretreatment with either NBQX (10 μM) or GYKI52466 (10 μM): maximal dendrite length [Two-way ANOVA interaction: $F(2,169) = 3.1$, $P<0.05$; treatment factor: $F(1,169) = 7.9$, $P<0.01$; inhibition factor: $F(2,169) = 5.2$, $P<0.01$], primary dendrite number [Friedman $(5,300) = 13.5$, $P<0.02$]

and soma area [Two-way ANOVA interaction: F(2,234) = 9.1, P<0.0005; treatment factor: F(1,234) = 21.4, P<0.0001; inhibition factor: F(2,234) = 8.9, P<0.0002]. **(h)** Cartoon representing intracellular pathway activation involved in structural plasticity following NMDAR blockade by either ketamine or Ro 25-6981 in mouse mesencephalic DA cultures. All data are expressed as mean values. In panels (**c, d**, **f** and **g**), values are represented as mean ± S.E.M. (***$P$<0.001, **$P$<0.01 vs. vehicle; $^{ooo}P$<0.001, $^{oo}P$<0.01, $^{o}P$<0.05 vs. ketamine, CX614 or Ro 25-6981, *post-hoc* Bonferroni's or Dunn's test). V = vehicle; K = ketamine; R = rapamycin; Ro = Ro 25-6981; GYKI = GYKI52466.

**Figure 3**. **BDNF-TrkB signaling is involved in structural plasticity induced by ketamine.** (**a**) Inhibition of the effects of ketamine on structural plasticity following pretreatment (20 min) with either an anti-BDNF blocking antibody (10 μg/ml) or a TrkB-Fc Chimera (5 μg/ml): maximal dendrite length [Two-way ANOVA interaction: F(2,174) = 11.5, P<0.0001; treatment factor: F(1,714) = 10.7, P<0.002; inhibition factor: F(2,174) = 2.9, ns], primary dendrite number [Friedman (5,300) = 12.7, P<0.05] and soma area [Two-way ANOVA interaction: F(2,234) = 8.4, P<0.0005; treatment factor: F(1,234) = 3.7, P<0.05; inhibition factor: F(2,234) = 11.4, P<0.0001]. (**b**) Inhibition of the effects of ketamine on structural plasticity following pretreatment (20 min) with either the TrkB phosphorylation inhibitor K252a, the Src phosphorylation inhibitor PP2 and the MEK phosphorylation inhibitor PD98059: maximal dendrite length [Two-way ANOVA interaction: F(3,232) = 10.7, P<0.0001; treatment factor: F(1,232) = 3.4, ns; inhibition factor: F(2,232) = 5.0, P<0.005], primary dendrite number [Friedman (7,400) = 18.1, P<0.02] and soma area [Two-way ANOVA interaction: F(3,312) = 13.8, P<0.0001; treatment factor: F(1,312) = 6.6,

P<0.01; inhibition factor: F(3,312) = 2.8, P<0.05]. (**c**) Inhibition of the effects of CX614 (10 μM) on structural plasticity following pretreatment (20 min) with either the anti-BDNF blocking antibody or the TrkB-Fc Chimera: maximal dendrite length [Two-way ANOVA interaction: F(2,167) = 6.9, P<0.002; treatment factor: F(1,167) = 15.1, P<0.0001; inhibition factor: F(2,167) = 8.1, P<0.001], primary dendrite number [Friedman (5,300) = 12.3, P<0.05] and soma area [Two-way ANOVA interaction: F(2,234) = 6.2, P<0.005; treatment factor: F(1,234) = 3.0, ns; inhibition factor: F(2,234) = 6.0, P<0.005]. All data are expressed as mean values ± S.E.M. (***$P$<0.001, **$P$<0.01 vs. vehicle; $^{ooo}P$<0.001, $^{oo}P$<0.01, $^{o}P$<0.05 vs. ketamine or CX614 *post-hoc* Bonferroni's or Dunn's test). V = vehicle; K = ketamine; α–BDNF = anti-BDNF blocking antibody; TrkB-Fc = TrkB-Fc Chimera; PD = PD98059.

**Figure 4**. **Permissive role of DA D3R in ketamine-induced structural plasticity in DA neurons.** (**a**) Inhibition of the effects of ketamine **(**1 μM) on structural plasticity following pretreatment (20 min) with the selective D3R antagonists SB277011-A (50 nM) and S33084 (10 nM): maximal dendrite length [Two-way ANOVA interaction: F(3,232) = 7.5, P<0.0001; treatment factor: F(1,232) = 35.5, P<0.0001; inhibition factor: F(3,232) = 1.7, ns], primary dendrite number [Friedman (7,400) = 24.0, P<0.001] and soma area [Two-way ANOVA interaction: F(3,312) = 5.2, P<0.002; treatment factor: F(1,312) = 14.2, P<0.0005; inhibition factor: F(3,312) = 2.7, P<0.05]. Pretreatment (20 min) with the D1R antagonist SCH23390 (1 μM) was ineffective. (**b**) Inhibition of the effects of CX614 (10 μM) on structural plasticity following pretreatment with SB277011-A (50 nM): maximal dendrite length [Two-way ANOVA interaction: F(1,116) = 22.7, P<0.0001; treatment factor: F(1,116) = 14.8, P<0.0002; inhibition factor: F(1,116) = 5.0, P<0.05], primary dendrite number

[Friedman (3,200) = 10.2, P<0.02] and soma area [Two-way ANOVA interaction: F(1,156) = 6.1, P<0.02; treatment factor: F(1,156) = 8.8, P<0.005; inhibition factor: F(1,156) = 5.6, P<0.002]. (**c)** Blockade of increase of p-p70S6K levels induced by 1 μM ketamine after pretreatment with SB277011-A (50 nM) analyzed by densitometry on normalized western blots: [Two-way ANOVA interaction: F(1,12) = 4.6, P<0.05; treatment factor: F(1,12) = 8.3, P<0.02; inhibition factor: F(1,12) = 3.8, ns]. **(d)** Representative photomicrographs of $TH^+$ neurons expressing p-p70S6K after vehicle**,** ketamine (1 μM)**,** pretreament with SB277011-A (50 nM) followed by ketamine (1 μM), or SB277011-A (50 nM) alone. (**e**) Semiquantitative image analysis of p-p70S6K fluorescence intensity. In $TH^+$ neurons, the significant increase of p-p70S6K is attenuated by SB277011-A pre-treatment [Two-way ANOVA interaction: F(1,116) = 11.0, P<0.002; treatment factor: F(1,116) = 21.1, P<0.0001; inhibition factor: F(1,116) = 19.3, P<0.0001]; in a sample of $TH^-$ neurons less inhibition by SB277011-A was observed [Two-way ANOVA interaction: F(1,238) = 3.3, ns; treatment factor: F(1,238) = 30.6, P<0.0001; inhibition factor: F(1,238) = 6.0, P<0.05]. Cell nuclei were stained with DAPI. Scale bar: 50 μm. (**f**) Structural plasticity in DA neurons from D3KO *vs* wild-type mice. Mesencephalic cultures were exposed to either ketamine (1 μM for 60 min), D3R agonist 7-OH-DPAT (10 nM) or BDNF (10 ng/ml) and structural plasticity was measured 72 hrs after treatment, showing prevalent significant genotype and treatment effects on maximal dendrite length [Two-way ANOVA interaction: F(3,232) = 4.76, P<0.005; treatment factor: F(3,232) = 17.32, P<0.0001; genotype factor: F(1,232) = 12.03, P<0.001], primary dendrite number [Friedman (7,400) = 30.5, P<0.0001] and soma area [Two-way ANOVA interaction: F(3,312) = 3.02, P<0.05; treatment factor: F(3,312) = 12.21, P<0.0001; genotype factor: F(1,312) = 9.98, P<0.002]. (**g**) Lack of effect of ketamine **(**1 μM) on p-

p70S6K levels in mesencephalic neuronal cultures from D3KO mice analyzed by densitometry on normalized western blots: [ANOVA main factor genotype: $F(1,24) = 44.42$, $P<0.0001$; main factor treatment: $F(2,24) = 6.31$, $P<0.01$; interaction: [$F(2,24) = 6.67$, $P<0.005$]. (**h**) Cartoon representing the molecular signaling involved in determining ketamine-induced structural plasticity in mouse mesencephalic DA cultures. In all panels, values are represented as mean ± S.E.M. (***$P<0.001$, **$P<0.01$, *$P<0.5$ vs. vehicle; °°°$P<0.001$, °°$P<0.01$, °$P<0.5$ vs. ketamine or CX614, *post-hoc* Bonferroni's or Dunn's test). V = vehicle; K = ketamine; SB = SB277011-A; S33= S33084; SCH= SCH23390; D3KO = D3R knockout mice; WT = wild-type mice.

**Figure 5**. **Structural plasticity in human iPSC-derived DA neurons *via* activation of mTOR pathway.** (**a**) Representative photomicrographs showing the morphological changes of human DA neurons exposed to vehicle, ketamine (1 μM for 60 min) or Ro 25-6981 (1 μM for 72 hrs). Cultures were fixed after 72 hrs and stained with an anti-TH antibody. Scale bar: 50 μm. (**b**) Inhibition of the effects of ketamine (1 μM) and Ro 25-6981 (1 μM) on structural plasticity following pretreatments (20 min) with NBQX (10 μM) or GYKI52466 (10 μM): maximal dendrite length [Two-way ANOVA interaction: $F(4,261) = 4.2$, $P<0.005$; treatment factor: $F(2,261) = 3.6$, $P<0.05$; inhibition factor: $F(2,261) = 12.4$, $P<0.0001$], primary dendrite number [Friedman $(8,450) = 26.8$, $P<0.001$] and soma area [Two-way ANOVA interaction: $F(4,351) = 3.5$, $P<0.01$, treatment factor: $F(2,351) = 2.1$, ns; inhibition factor: $F(2,351) = 10.4$, $P<0.0001$]. (**c**) Inhibition of the effects of ketamine on structural plasticity following pretreatment (20 min) with either an anti-BDNF blocking antibody (10 μg/ml) or a TrkB-Fc Chimera (5 μg/ml): maximal dendrite length [Two-

way ANOVA interaction: $F(2,174) = 4.1$, $P<0.02$; treatment factor: $F(1,174) = 1.7$, ns; inhibition factor: $F(2,174) = 2.8$, ns], primary dendrite number [Friedman $(5,300) = 13.7$, $P<0.02$] and soma area [Two-way ANOVA interaction: $F(2,234) = 4.3$, $P<0.02$; treatment factor: $F(1,234) = 3.3$, ns; inhibition factor: $F(2,234) = 3.9$, $P<0.05$]. (**d**) Inhibition of the effects of ketamine on structural plasticity following pretreatment (20 min) with either the TrkB phosphorylation inhibitor K252a, the Src phosphorylation inhibitor PP2 and the MEK phosphorylation inhibitor PD98059: maximal dendrite length [Two-way ANOVA interaction: $F(3,232) = 4.3$, $P<0.002$; treatment factor: $F(1,232) = 1.5$, ns; inhibition factor: $F(3,232) = 3.2$, $P<0.05$], primary dendrite number [Friedman $(7,400) = 15.0$, $P<0.05$] and soma area [Two-way ANOVA interaction: $F(3,312) = 3.3$, $P<0.02$; treatment factor: $F(1,312) = 1.8$, ns; inhibition factor: $F(3,312) = 3.9$, $P<0.01$]. (**e**) Inhibition of effects of ketamine (1 μM) by pretreatment (20 min) with SB277011-A (50 nM), S33084 (10 nM), SCH23390 (1 μM): maximal dendrite length [Two-way ANOVA interaction: $F(3,232) = 5.0$, $P<0.005$; treatment factor: $F(1,232) = 20.5$, $P<0.0001$; inhibition factor: $F(3,232) = 2.7$, $P<0.05$], primary dendrite number [Friedman $(7,400) = 22.0$, $P<0.005$] and soma area [Two-way ANOVA interaction: $F(3,312) = 4.3$, $P<0.01$; treatment factor: $F(1,312) = 7.2$, $P<0.01$; inhibition factor: $F(3,312) = 3.0$, $P<0.05$]. (**f**) Inhibition of effects of ketamine (1 μM) by pretreatment (20 min) with LY294002 (10 μM) or rapamycin (20 nM): maximal dendrite length [Two-way ANOVA interaction: $F(2,174) = 3.8$, $P<0.05$; treatment factor: $F(1,174) = 3.0$, ns; inhibition factor: $F(2,174) = 5.3$, $P<0.01$], primary dendrite number [Friedman $(5,300) = 13.2$, $P<0.05$] and soma area [Two-way ANOVA interaction: $F(2,234) = 5.4$, $P<0.005$; treatment factor: $F(1,234) = 0.8$, ns; inhibition factor: $F(2,234) = 2.0$, ns]. (**g**) Effects of transient exposure (2-60 min) to 1 μM ketamine on p-p70S6K levels analzyed by densitometry

on normalized western blots: [One-way ANOVA $F(5,18) = 3.10$, $P<0.05$]; p-p70S6K levels were normalized to the corresponding p70S6K, TH and tubulin levels. The densitometric values are represented as percentage of vehicle values. Maximal increase was seen at 5 min after ketamine exposure. (**h**) Representative photomicrographs of $TH^+$ neurons (red) expressing p-p70S6K (green) after vehicle or 1 μM ketamine. (**i**) Semiquantitative image analysis of p-p70S6K fluorescence intensity in $TH^+$ and $TH^-$ neurons: ketamine increased p-p70S6K levels *vs* vehicle in $TH^+$ [$t(68) = 9.4$, $P<0.0001$] and $TH^-$ [$t(138) = 7.6$, $P<0.0001$]. Cell nuclei were stained with DAPI. Scale bar: 50 μm. In all panels, values are represented as mean ± S.E.M. (***$P<0.001$, **$P<0.01$, *$P<0.5$ vs. vehicle; °°°$P<0.001$, °°$P<0.01$, °$P<0.5$ vs. ketamine or Ro 25-6981; *post-hoc* Bonferroni's or Dunn's test).

**Table 1.** Effects of (2R,6R)-HNK on structural plasticity in mouse mesencephalic DA neurons.

| | Mouse mesencephalic DA neurons | | | | | |
|---|---|---|---|---|---|---|
| | Dendrite length (µm) | | Dendrite number | | Soma area (µm$^2$) | |
| | 60 min | 6 hrs | 60 min | 6 hrs | 60 min | 6 hrs |
| **Vehicle** | 95.08±3.41 | 98.25±2.93 | 1.38±0.08 | 1.46±0.09 | 96.03±2.29 | 98.28±3.11 |
| **0.1 µM** | 103.80±4.40 | 105.40±3.20 | 1.68±0.09 | 1.82±0.09 | 101.30±4.08 | 110.80±2.99 |
| **0.5 µM** | 112.70±5.02* | 123.80±4.95*** | 1.70±0.10* | 1.94±0.11** | 110.90±3.99* | 121.10±4.15*** |

Statistical analysis: 60 min exposure, maximal dendrite length [One-way ANOVA $F(2,87) = 4.14$; $P<0.05$], primary dendrite number [Kruskal-Wallis $(2,450) = 7.6$, $P<0.05$] and soma area [One-way ANOVA $F(2,117) = 4.50$; $P<0.05$]; 6 hrs exposure, maximal dendrite length [One-way ANOVA $F(2,87) = 12.00$; $P<0.0001$], primary dendrite number [Kruskal-Wallis $(2,450) = 13.2$, $P<0.002$] and soma area [One-way ANOVA $F(2,117) = 10.90$; $P<0.0001$]. Values are expressed as mean ± S.E.M. (***$P<0.001$; **$P<0.01$; *$P<0.05$ vs. vehicle; *post-hoc* Bonferroni's or Dunn's test).

**Table 2.** Effects of (2R,6R)-HNK on structural plasticity in human iPSC-derived DA neurons.

| | Human iPSC-derived DA neurons | | | | | |
|---|---|---|---|---|---|---|
| | Dendrite length (µm) | | Dendrite number | | Soma area (µm$^2$) | |
| | 60 min | 6 hrs | 60 min | 6 hrs | 60 min | 6 hrs |
| **Vehicle** | 113.30±3.08 | 112.40±3.02 | 1.46±0.09 | 1.48±0.08 | 118.40±2.74 | 117.90±2.35 |
| **0.1 µM** | 119.20±4.15 | 121.90±3.65 | 1.66±0.11 | 1.68±0.10 | 123.60±3.70 | 125.40±3.47 |
| **0.5 µM** | 128.10±3.57* | 132.0±3.86*** | 1.90±0.11* | 2.02±0.12** | 132.90±4.93* | 136.80±3.85*** |

Statistical analysis: 60 min exposure, maximal dendrite length [One-way ANOVA $F(2,87) = 4.21$; $P<0.05$], primary dendrite number [Kruskal-Wallis $(2,450) = 8.5$, $P<0.02$]and soma size [One-way ANOVA $F(2,117) = 3,54$; $P<0.05$]; 6 hrs exposure, maximal length of dendrites [One-way ANOVA $F(2,87) = 7.75$; $P<0.0001$], number of primary dendrites [Kruskal-Wallis $(2,450) = 11.6$, $P<0.005$] and soma area [One-way ANOVA $F(2,117) = 8.39$; $P<0.001$]. Values are expressed as mean ± S.E.M. (***$P<0.001$; **$P<0.01$; *$P<0.05$ vs. vehicle; *post-hoc* Bonferroni's or Dunn's test).